Attachment I – Protocol

Ecolab
Study Identification Number 1400050

REGULATED PESTICIDE EFFICACY STUDY PROTOCOL

STUDY TITLE: KX-6228 Non-Food Contact Sanitizing Efficacy

EPA REG. NO.: 1677-

ECOLAB GLP STUDY NUMBER: 1400050

PROPOSED STUDY INITIATION/COMPLETION DATES

Initiation **April 21, 2014**

Completion **July 1, 2014**

DESCRIPTION OF STUDY OBJECTIVE

KX-6228 (EPA Registration No. 1677-) will be tested to determine non-food contact surface sanitizing efficacy against *Staphylococcus aureus* ATCC 6538 and *Enterobacter aerogenes* ATCC 13048 with the test parameters outlined below. ASTM Method E1153-03 (reapproved 2010) was the test method utilized in making the sanitizing claim.

Test Parameters

Ecolab SOP number:	MS016-26; *Non-Food Contact Sanitizer Test Method*
Test System:	*Staphylococcus aureus* ATCC 6538 *Enterobacter aerogenes* ATCC 13048
Organic Soil Load:	5% Fetal Bovine Serum
Exposure Time:	5 minutes
Exposure Temperature:	Ambient (15-30°C)
Test Substance Batches:	P081931 P111431 P120331
Test Substance Diluent:	500 ppm synthetic hard water
Test Substance Concentration:	The test substance will be diluted at 1 oz/8 gallons to result in the active ingredients at or below the lower limits of 114 ppm hydrogen peroxide, 23.9 ppm peroxyacetic acid (POAA) and 5.74 ppm peroxyoctanoic acid (POOA)

TEST SUBSTANCE IDENTIFICATION

Test Substance Name: **KX-6228**

Alternate Test Substance Names: Spartan

Batch Identification:

1. P081931
2. P111431
3. P120331

All batches aged ≥60 days.
Use-solution chemical quality verification performed on batch P120331 under Ecolab GLP study number 1400009.

Formula Code: Pending

Date of Manufacture:

KX-6228 Batch Identification	Date of Manufacture
P081931	08/19/13
P111431	11/14/13
P120331	12/03/13

An aliquot of the test substance will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, MN until the quality of the formula no longer affords evaluation. Test substance not dispersed for retention, chemical quality verification or efficacy testing will be stored in Ecolab Microbiological Services cabinet until disposed.

QUALITY ASSURANCE UNIT MONITORING

The protocol, pesticide efficacy in-life and final report are proposed to be inspected by the Ecolab Quality Assurance Unit (QAU) in accordance with their current standard operating procedures. The following proposed Ecolab QA inspections are for planning purposes only and may change. Ecolab QA inspections that are performed, along with their dates and auditors, will be included in the study final report. Changes in Ecolab QA inspections from those proposed below will not require revision of this protocol.

Proposed QAU Monitoring

Protocol Audit
Pesticide Efficacy In-Life Inspection
Final Report Audit

CHEMICAL QUALITY VERIFICATION

Proposed Experimental Initiation/Termination Dates

The chemical quality verification on the test substance concentrate batch P081931 was performed under Ecolab GLP study 1300148. The chemical quality verification on the test substance concentrate batches P111431 and P120331 were performed under Ecolab GLP study 1300150. The chemical quality verification on the single batch of test substance use-solution was performed under Ecolab GLP study number 1400009. Initiation and termination dates are documented within those studies.

Method

Chemical analysis was performed on each batch of test substance concentrate to determine the concentration of the active ingredients. The chemical analysis was conducted under Ecolab GLP study number 1300148 (batch P081931) and Ecolab GLP study number 1300150 (batch P111431 and batch P120331). Chemical analysis was performed on a single batch of test substance use-solution conducted under Ecolab GLP study number 1400009. The test substance use-solution was prepared with batch P120331 by adding 1.57 ± 0.03 g test substance with 1498.43 ± 0.03 g of laboratory purified water to achieve a 1 oz/ 8 gallons dilution of formula.

The following calculations were used to determine the amount of test substance in a 1,500 g use-solution at a dilution of 1 oz/ 8 gallon to result in a use-solution at or below the lower limits of 114 ppm hydrogen peroxide, 23.9 ppm peroxyacetic acid and 5.74 ppm peroxyoctanoic acid:

% Dilution = (1 oz/8 gallons) (1 gallon/128 oz) (100%) = 0.0977%

ppm at the lower limit = (% LCL/100) (% Dilution/100) (specific gravity) 10^6 =
ppm at the lower limit for hydrogen peroxide =
(9.75/100) (0.0977/100) (1.20) 10^6 = 114 ppm
ppm at the lower limit for peroxyacetic acid =
(2.04/100) (0.0977/100) (1.20) 10^6 = 23.9 ppm
ppm at the lower limit for peroxyoctanoic acid =
(0.49/100) (0.0977/100) (1.20) 10^6 = 5.74 ppm

Amount of test substance in a 1,500 g use-solution at a dilution of 1 oz/8 gallon to result in a use-solution at or below the lower limits of 114 ppm hydrogen peroxide, 23.9 ppm peroxyacetic acid and 5.74 ppm peroxyoctanoic acid =

$$\frac{(\text{ppm Active at LCL})(1{,}500\text{g})(100)}{(\%\ \text{Active from Analysis})\ (10^6)}$$

To ensure the test substance use-solution is at or below the lower limits of 114 ppm hydrogen peroxide, 23.9 ppm peroxyacetic acid and 5.74 ppm peroxyoctanoic acid, the test substance use-solution preparation was based on the concentration of the peroxyacetic acid as shown in the table below.

KX-6228 Batch Identification	% Active in Concentrate from Analysis*	Amount of Test Substance: Amount of Diluent
P120331	Hydrogen Peroxide = 10.72%	1.60 g ± 0.03 g : 1498.40 ± 0.03 g
	Peroxyacetic Acid = 2.29%	**1.57 g ± 0.03 g : 1498.43 ± 0.03 g**
	Peroxyoctanoic Acid = 0.50%	1.72 g ± 0.03 g : 1498.28 ± 0.03 g

*Results determined under Ecolab GLP study number 1400009 on 03/03/14.

The chemical quality verification was performed by the Analytical Lab using the methods listed below. The methods have been deemed acceptable by the Analytical Lab and the study sponsor to ensure proper characterization of the test substance concentrate and test substance use-solution. Statistical treatment of test results may be inherent to the method. Additional volumes and dilutions may be necessary to determine the chemistry of the use-solution sample.

QATM-202B was used for hydrogen peroxide analysis in both the use-solution and concentrates. QATM-317 was used for total peracid analysis in the use-solution. QATM-337 was used for the peroxyacetic acid and peroxyoctanoic acid analysis in the concentrates.

QATM-202B; *Hydrogen Peroxide and Peracid Analysis by Titration with Potassium Permanganate*

Hydrogen peroxide content is determined by an oxidation-reduction titration with potassium permanganate. After the endpoint of this titration has been reached, an excess of potassium iodide is added to the solution. The potassium iodide reacts with peracids to liberate iodine, which is titrated with a standard solution of sodium thiosulfate.

QATM-317; *Suppressed Peroxide Titration for Peracids and Hydrogen Peroxide*

The method requires two steps for the determination of peroxyacetic acid (POAA) and peroxyoctanoic acid (POOA). The first step determines the POAA content by filtering out POOA and persulfonated oleic acid (PSOA) while suppressing the hydrogen peroxide by cold temperatures. The presence of deionized ice in the reaction flask does not interfere with the titration chemistry.

The second step rinses the POOA and PSOA off of the filter with solvent. The PSOA is precipitated using calcium acetate and filtered out of the solution. The POOA can then be measured.

QATM-337; *Peroxyacetic Acid and Peroxyoctanoic Acid Determination by Thiosulfate Titration*

The method requires two steps for the determination of peroxyacetic acid (POAA) and peroxyoctanoic acid (POOA). The first step determines the POAA content by filtering out POOA and persulfonated oleic acid (PSPA, if present) while suppressing the hydrogen peroxide by cold temperature. The presence of deionized ice in the reaction flask does not interfere with the titration chemistry.

The second step rinses the POOA and PSOA (if present) off of the filter with solvent. The PSOA is precipitated using calcium acetate and filtered out of the solution. The POOA can then be measured.

The most current QATMs were used during the course of this study for the chemical and physical analysis.

Interpretation of Results

The concentration of the active ingredient in the test substance concentrates will be judged acceptable for pesticide efficacy testing if within the ranges specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

Active Ingredients	CSF Lower Certified Limit	CSF Upper Certified Limit
Hydrogen Peroxide	9.75	11.55
Peroxyacetic Acid	2.04	2.72
Peroxyoctanoic Acid	0.49	0.78

The concentration of the active ingredients in the test substance use-solution will be judged acceptable for pesticide efficacy testing if the actives meet the acceptance criteria determined in the chemical quality verification performed under Ecolab GLP study number 1400009. The concentration acceptance criteria are shown in the table below.

Active Ingredients	1 oz/8 gallon Use-Solution Acceptance Criteria
Hydrogen Peroxide	≤0.0115% or ≤115 ppm
Total Peracid	≤0.0029% or ≤29 ppm

The Chemical Quality Verification results will be reported in the final report of this study.

PESTICIDE EFFICACY TESTING

Proposed Experimental Start/Termination Dates

Experimental Start Date	April 2014
Experimental Termination Date	April 2014

Methods

Pesticide efficacy data will be generated by the Microbiology Lab using the most current methods listed below. See the specific methods in the Protocol Appendix.

Method Number	Method Name
MS002-17	*Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims*
MS008-24	*Synthetic Hard Water Preparation & Standardization*
MS016-26	*Non-Food Contact Sanitizer Test Method*
MS088-19	*Test Substance Use-Solution Preparation for Analysis*

Test Method Requirement and Test System Justification

The following apply when determining the effectiveness of a non-food contact surface sanitizer; 5 carriers are required on each of three samples, representing different batches one of which is greater than 60 days old. The required organisms are *Staphylococcus aureus* ATCC 6538 and *Enterobacter aerogenes* ATCC 13048. ASTM Method E1153-03 (reapproved 2010), Standard Test Method for Efficacy of Sanitizers Recommended for Inanimate Non-Food Contact Surfaces, for the above stated organisms are recommended based on the U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2300 Sanitizers for Use on Hard Surfaces –Efficacy Data Recommendations September 4, 2012. Also, U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2000 General considerations for Public Health Uses of Antimicrobial Agents September 4, 2012 applies to this study.

Test Method Justification

Ecolab Microbiological Services SOP MS016-26; *Non-Food Contact Sanitizer Test Method* will be the test method utilized in this study.

Test Systems and Identification

The test systems which will be utilized for this procedure *Staphylococcus aureus* ATCC 6538 and *Enterobacter aerogenes* ATCC 13048. Identification will be performed by observing the colony morphology and performing a Gram stain.

Organic Soil

5% Fetal Bovine Serum

Statement of Proposed Statistical Method

None

Test Substance Diluent

500 ppm Synthetic Hard Water prepared as described in Ecolab Microbiological Services SOP MS008-24; *Synthetic Hard Water Preparation & Standardization* will be the diluent.

Test Substance Concentration

Antimicrobial efficacy testing will be performed with the test substance diluted at 1 oz/8 gallon to result in a use-solution at or below the lower limits of 114 ppm hydrogen peroxide, 23.9 ppm peroxyacetic acid and 5.74 ppm peroxyoctanoic acid.

% Dilution = (1 oz/8 gallons) (1 gallon/128 oz) (100%) = 0.0977%

ppm at the lower limit = (% LCL/100) (% Dilution/100) (specific gravity) 10^6 =

ppm at the lower limit for hydrogen peroxide =
(9.75/100) (0.0977/100) (1.20) 10^6 = 114 ppm

ppm at the lower limit for peroxyacetic acid =
(2.04/100) (0.0977/100) (1.20) 10^6 = 23.9 ppm

ppm at the lower limit for peroxyoctanoic acid =
(0.49/100) (0.0977/100) (1.20) 10^6 = 5.74 ppm

Amount of test substance in a 1,500 g use-solution at a dilution of 1 oz/8 gallon to result in a use-solution at or below the lower limits of 114 ppm hydrogen peroxide, 23.9 ppm peroxyacetic acid and 5.74 ppm peroxyoctanoic acid =

$$\frac{(\text{ppm Active at LCL})(1{,}500\text{g})(100)}{(\%\ \text{Active from Analysis})\ (10^6)}$$

To ensure the test substance use-solution is at or below the lower limits of 114 ppm hydrogen peroxide, 23.9 ppm peroxyacetic acid and 5.74 ppm peroxyoctanoic acid, the test substance use-solution preparation was based on the concentration of the peroxyacetic acid as shown in the table below.

Batch Identification	% Active from Analysis	Desired ppm Active	Test Substance Weight: Diluent Weight (g)
P081931[a]	10.60% Hydrogen Peroxide	114 ppm Hydrogen Peroxide	1.61 g : 1498.39 g
	2.29% Peroxyacetic Acid	**23.9 ppm Peroxyacetic Acid**	**1.57 g : 1498.43 g**
	0.52% Peroxyoctanoic Acid	5.74 ppm Peroxyoctanoic Acid	1.66 g : 1498.34 g
P111431[b]	10.4% Hydrogen Peroxide	114 ppm Hydrogen Peroxide	1.64 g : 1498.36 g
	2.3% Peroxyacetic Acid	**23.9 ppm Peroxyacetic Acid**	**1.56 g : 1498.44 g**
	0.53% Peroxyoctanoic Acid	5.74 ppm Peroxyoctanoic Acid	1.62 g : 1498.38 g
P120331[b]	10.5% Hydrogen Peroxide	114 ppm Hydrogen Peroxide	1.63 g : 1498.37 g
	2.3% Peroxyacetic Acid	**23.9 ppm Peroxyacetic Acid**	**1.56 g : 1498.44 g**
	0.53% Peroxyoctanoic Acid	5.74 ppm Peroxyoctanoic Acid	1.62 g : 1498.38 g

[a] % Active from Analysis determined on 01/08/14-01/09/14 under Ecolab GLP study number 1300148.
[b] % Active from Analysis determined on 04/01/14 under Ecolab GLP study number 1300150.

Note: The % Active from Analysis concentrations for batch P120331 was determined on 04/01/14 under Ecolab GLP study number 1300150. The test substance dilution procedure did change from when based on the % Active from Analysis concentrations determined on 03/03/14 under Ecolab GLP study number 1400009 which were the results the use-solution chemical quality verification was based on. The use-solution for the chemical quality verification was prepared with 1.57 ± 0.03 g test substance and 1498.43 ± 0.03 g diluent. The use-solution for efficacy testing will target 0.01 grams less of the test substance based on the most recent % Active from Analysis concentrations which is a worse-case scenario.

The weights of the test substance and diluent can be +/- 0.03 g of the weight given in the above table.

Test Surface

Stainless Steel, non-corrosive, 25 x 25 mm (1 x 1")

Exposure Time/Temperature

The test systems will be exposed to the test substance for 5 minutes at ambient temperature (15 – 30 °C).

Neutralizer Medium

DE Broth

Plating Medium

Tryptone Glucose Extract Agar

Incubation Time/Temperature

S. aureus ATCC 6538 plates will be incubated for 48 ± 4 hours at 35 ± 2°C.
E. aerogenes ATCC 13048 plates will be incubated for 48 ± 4 hours at 30 ± 2°C.

Test Controls

The following controls will be incorporated with the test procedure for each test system:

a. Inoculum Count
b. Inoculum Numbers Control Squares
c. Neutralization Controls
d. Test Substance Diluent Sterility Control
e. Organic Soil Sterility Control
f. Test System Purity

Details on each of the above controls can be found in Ecolab SOP MS016-26 located in Protocol Appendix.

Interpretation of Test Results

The performance standard for a non-food contact sanitizer is ≥99.9% reduction in the numbers of both *Staphylococcus aureus* ATCC 6538 and *Enterobacter aerogenes* ATCC 13048 compared to the inoculum numbers control square results.

DATA RETENTION

Following the completion of the study, the original raw data and final report will be archived at the Ecolab Schuman Campus in Eagan, Minnesota or at an approved off-site location. All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained for the life of the commercial product plus four years.

TEST SUBSTANCE RETENTION

An aliquot of each batch of test substance will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, Minnesota until the quality of the formula no longer affords evaluation.

GOOD LABORATORY PRACTICES

This study will be conducted according to Good Laboratory Practices, as stated in 40 CFR Part 160. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. The sponsor will be notified as soon as it is practical whenever an event occurs that could have an effect on the validity of the study.

- **Name and Address of Sponsor**

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4/21/2014
Date

Laurinda Holen
Study Director

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Date